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14. ABSTRACT
Previously, that the cell adhesion molecule ß-catenin forms a complex with the androgen receptor (AR) and modulate itstranscription. The cross talk between ß-catenin and AR signaling can play an important role in AR transcriptional in prostatecancer progression. Our preliminary data seem indicate stromally derived paracrine Wnt family members activate theepithelial frizzled receptor to enable prostate epithelial survival in an androgen deficient environment. We will continue totest the original hypothesis that there is a direct molecular interaction between ß-catenin and the C-terminus region of ARinvolved in the mechanism of prostate androgen responsiveness. The physiologic response to androgen ablation(castration) differ significantly between the prostatic stroma and epithelia despite the common expression of ß-catenin andAR, as evidence for the different transcriptional cofactor interactions found in prostatic epithelial and stromal cells. Ourresults support the data that show ß-catenin mutations enabling ß-catenin stability in prostate cancer can lead to androgenindependence. At least two genetic interventions for hormonally refractive prostate cancer have emerged from our work: 1) the expression of Hic-5 in the prostatic epithelia to inhibit canonical Wnt signaling and 2) antagonism of Wnt receptor/ligandinteraction such as the expression of SFRP-2.

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None provided.

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INTRODUCTION

The mortality due to prostate cancer primarily results from the failure of androgen-ablation therapy and metastasis to distant sites. New treatments are required that are more effective irrespective of the structure of the AR in the cancerous epithelial cells. Previously the oncogene ß-catenin has been shown to be mutated in human prostate cancer [1, 2]. ß-Catenin has a role in cell-cell adhesion and is an essential signaling molecule in the Wnt signaling pathway where it acts as a transcriptional coactivator for the T cell factor (TCF) family of proteins [3, 4]. The canonical Wnt signaling is associated with elevated proliferation and neoplastic transformation of various epithelial tissues [5]. The cross talk between ß-catenin and AR signaling can play a role in prostate cancer progression [6-9]. The original objective of this proposal was to determine the mechanism by which ß-catenin modulates AR signaling. However due to the recent intensity of research in this field and research delays from transferring the grant from Dr. Cristina Truica (original PI) to the current PI, the proposal outlook has been broadened.

Transforming growth factor beta (TGF-ß) is up-regulated in the prostate immediately following androgen ablation. The role of TGF-ß in on the prostate is thought to be as a mediator of apoptosis, as supported by its coincident upregulation and downstream signaling with prostatic regression in benign and cancer tissues {Kyprianou, 1988 #43}. TGF-ß signals through binding of cell surface TGF-ß type II receptor that in turn recruits the TGF-ß type I receptor for downstream activation of cytoplasmic proteins, prominently Smad2 and Smad3. TGF-ß acts generally to suppress proliferation in the prostatic epithelia and is negatively regulated by androgens. It has been previously reported that the TGF-ß and the androgen receptor (AR) pathway act together to regulate prostatic proliferation [10]. However, the TGF-ß, androgen cross-talk is not isolated to intracellular signaling, but likely involve autocrine and paracrine actions of multiple growth factors.

Reported observations of the PI show that a mouse model lacking transforming growth factor beta (TGF-ß) signaling in the stromal compartment spontaneously develops pre-neoplastic lesions in the prostate [11]. More recent unpublished data suggest that the prostate tissue the same mouse model is insensitive to androgen ablation and has altered ß-catenin activation compared to normal mice. Thus the cross-talk among the ß-catenin, androgen signaling, and TGF-ß signaling pathways have a potentially important role as a whole in prostate androgen responsiveness. Further, the mouse model suggests, the prostatic stromal fibroblasts are critical to epithelial androgen signaling. Dr. Truica had found that the ß-catenin, with a complex of other proteins that she has identified, could interact with the androgen receptor and effect DNA binding. We tested the original hypothesis that there is a direct molecular interaction between ß-catenin and the C-terminus region of AR involved in the mechanism of prostate androgen responsiveness. However, we further examined the repercussions of the interaction in both LNCaP (originally proposed) and mouse prostatic stromal cells. The physiologic response to androgen ablation differ significantly between the prostatic stroma and epithelia despite the common expression of ß-catenin and AR, as evidence for the different transcriptional cofactor interactions found in prostatic epithelial and stromal cells. Ultimately the paracrine signaling in vivo between the stromal and epithelial compartments dictate the androgen responsiveness of the tissue.

BODY

The approved statement of work comprised of three main tasks:

Task 1. Investigate the effects of loss-of-function of ß-catenin on the LNCaP prostate epithelial cells

Task 2. Mechanisms involved in the development of androgen independent prostate cancer.

Task 3. Determine if E-cadherin and AR compete to binding the same domain of ß-catenin.

The results for each task will be described in this report.

Task 1

The loss of ß-catenin function can be in the form of its degradation/neutralization or a mutation that results in its inability to be degraded. The latter condition is most commonly described in many cancer types including prostate cancer. Not surprisingly, the two forms of ß-catenin loss of function have very different roles on androgen sensitivity of the prostate epithelia. Preliminary data for this project, corroborated by reports of others, indicate ß-catenin can bind and regulate androgen receptor. We and others have shown that expression of the degradation-resistant ß-catenin (having a mutation in the phosphorylation site Serine 33) results in elevated AR activity on androgen responsive genes. Further anti-androgen, bicalutamide, treatment inhibits AR and ß-catenin loading of the PSA enhancer element, but has little effect on the promoter, ARE1 (androgen response element 1) based on ChIP (chromatin immunoprecipitation) analysis. AR binds ß-catenin through a LXXLL amino acid motif. A similar LXXLL motif found in the p160/SRC transcription factor is reported to support steroid receptor transcriptional activity. Since ß-catenin couples to AR in a complex with (T-cell factor 4) TCF4, we wanted to investigate whether the AR - p160/SRC complex also involve TCF4. Immunoprecipitation results supported that TCF4 is part of the AR-p160/SRC complex in the presence of DHT. This would imply that TCF4 is not exclusively a canonical Wnt signaling protein, and opens up the possibility an alternative drug target to inhibit androgen non-responsive prostate cancer that would also inhibit those cancers with ß-catenin mutations.

The interaction of the ß-catenin and AR, conversely also affects ß-catenin responsive genes. Such genes include the activation of c-myc and cyclinD1 through the canonical Wnt signaling pathway. Examination of ß-catenin transcriptional activity on the TCF binding site show that AR expression has little effect alone, but both DHT and bicalutamide could inhibit TCF transcriptional activity (Figure 1). Independent of AR-specific transcriptional activities the regulation of ß-catenin signaling through by the AR-DHT or AR-bicalutamide complex suggest another mechanism by which AR-independent prostate cancer differ AR-dependent prostate tissue. As evidence, the mutation of the C-terminal AR position, V716R, was unable to suppress TCF transcriptional activity through ß-catenin binding. Thus there is a counter relationship between AR and ß-catenin where AR inhibits ß-catenin transcriptional activity, and ß-catenin stimulates AR transcriptional activity.

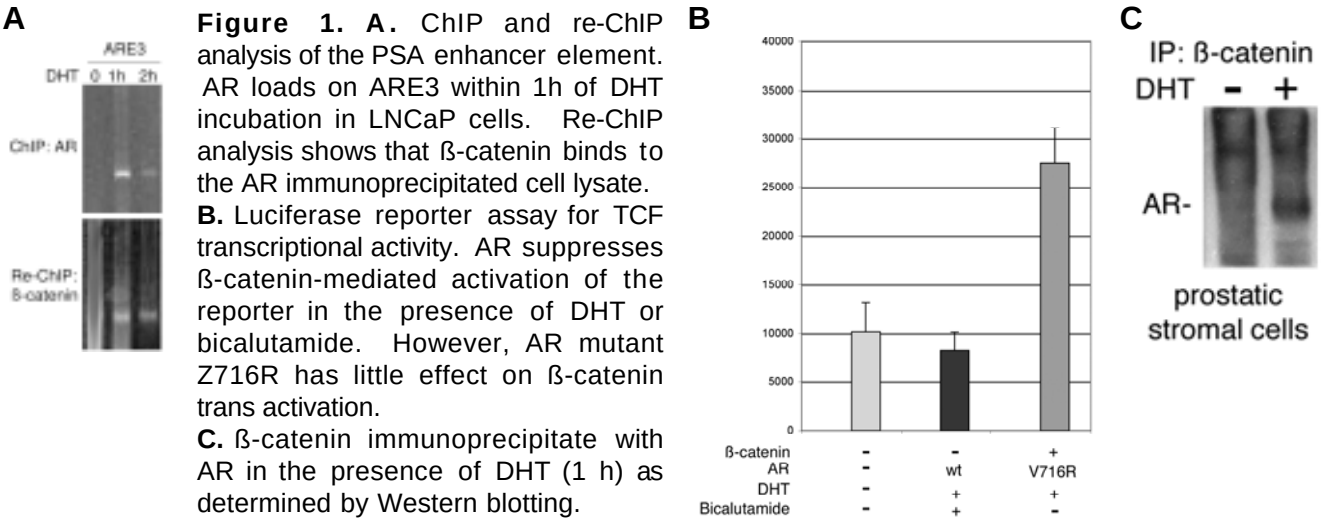

Figure 1. A. ChIP and re-ChIP analysis of the PSA enhancer element. AR loads on ARE3 within 1h of DHT incubation in LNCaP cells. Re-ChIP analysis shows that ß-catenin binds to the AR immunoprecipitated cell lysate. **B.** Luciferase reporter assay for TCF transcriptional activity. AR suppresses ß-catenin-mediated activation of the reporter in the presence of DHT or bicalutamide. However, AR mutant Z716R has little effect on ß-catenin trans activation. **C.** ß-catenin immunoprecipitate with AR in the presence of DHT (1 h) as determined by Western blotting.

Task 2

Our previous studies suggest the loss of TGF-ß responsiveness in the prostatic stromal cells results in adjacent epithelia that is refractive to androgen ablation in transgenic mouse models (supported by NIH CA108646 and DOD W81XWH-04-1-0046 grants). To determine the mechanism of the role of stromal TGF-ß signaling on the prostate gland we chose to use a simpler experimental system. Initially, the cultured prostatic stromal cells from Tgfbr2$^{floxE2/floxE2}$ (control) and Tgfbr2^{fspKO} (stromal knock for the TGF-ß receptor type II) mice and verified the loss of TGF-ß responsivity in Tgfbr2^{fspKO} cultures [11]. Immunolocalization of Smad 2, 3 showed nuclear translocation in Tgfbr2$^{floxE2/floxE2}$ stromal cultures upon TGF-ß1 treatment, but such Smad translocation was nearly absent in Tgfbr2^{fspKO} stromal cultures under similar conditions (Figure 2).

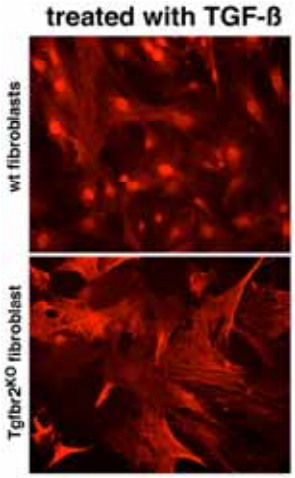

Figure 2. Smad2,3 immunolocalization in prostatic stromal cells from control and Tgfbr2^{fspKO} mice following 6h treatment with TGF-ß (5 ng/ml).

The proliferative rates of the Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} stromal cells were determined by thymidine incorporation assays. Figure 3 illustrates a two fold higher proliferative rate of the Tgfbr2^{fspKO} stroma compared to the control, Tgfbr2$^{floxE2/floxE2}$ cells. Further treatment with the androgen receptor antagonist, bicalutamide, decreased thymidine incorporation of the respective Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} stromal proliferation by approximately 20%. Under similar conditions, there was negligible proliferative response to bicalutamide treatment by prostatic epithelia with mutated androgen receptor, PC3 and RWPE-1 cells. LNCaP cells, with intact androgen receptor expression, exhibited nearly 80% decrease in proliferation in response to bicalutamide compared to vehicle treatment. Thus, LNCaP cells were used in the subsequent experiments to examine the paracrine effects of stromal cells on androgen responsiveness.

Stromal conditioned media was incubated with LNCaP cells for determining stromally derived paracrine factors that influence androgen responsivity. Tgfbr2$^{floxE2/floxE2}$-conditioned media inhibited the proliferation of the LNCaP cells, while not surprisingly Tgfbr2^{fspKO}-conditioned media stimulated its proliferation, as determined through thymidine incorporation assays (Figure 3). It was necessary to distinguish the role of androgen ablation in the stroma verus the epithelia that contributed to epithelial proliferation. First, conditioned media obtained from stromal cells treated with bicalutamide were incubated with the LNCaP cells. Bicalutamide treated Tgfbr2$^{floxE2/floxE2}$ conditioned media resulted in a sixteen-fold greater LNCaP proliferation than Tgfbr2$^{floxE2/floxE2}$ conditioned media alone. Similarly, bicalutamide treated, Tgfbr2^{fspKO}-conditioned media resulted in greater LNCaP proliferation compared to Tgfbr2^{fspKO} conditioned media alone. Note, bicalutamide incubation at 37ºC for three days (cell-free), resulted in similar androgen receptor antagonistic activity on the target LNCaP cells as fresh bicalutamide treatment. However, when we treated the LNCaP cells with Tgfbr2$^{floxE2/floxE2}$-conditioned media and added bicalutamide to the conditioned media, low proliferation was observed regardless of the source of stromal media, similar to bicalutamide treatment of LNCaP cells alone. If Tgfbr2^{fspKO}-conditioned media was replaced under similar conditions the LNCaP cell proliferation was elevated (Figure 3). This suggested that both Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} stromal cells express a factor that supports epithelial proliferation in response to androgen receptor antagonism. However, the TGF-ß-insensitive stroma seems to produce this factor constitutively. These experiments isolated the role of androgen ablation on the stromal and epithelial compartments to suggest a proliferative stromally derived paracrine factor.

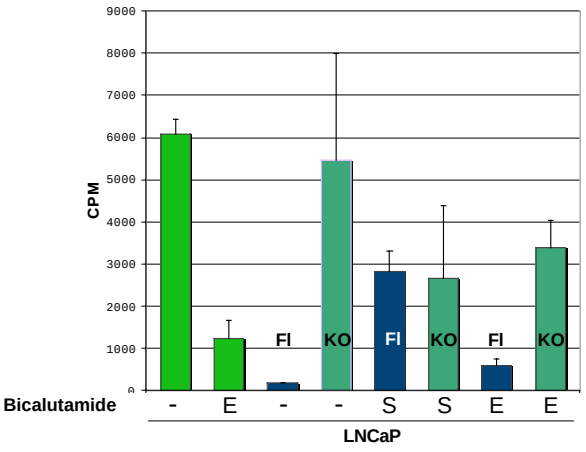

Figure 3. Thymidine incorporation assays of LNCaP cells in the presence or absence of conditioned media from Tgfbr2$^{floxE2/floxE2}$ or Tgfbr2^{fspKO} cells. Bicalutamide treatment was performed on the stromal cells (S) or LNCaP epithelial cells (E).

Epithelial Wnt signaling has been suggested as a potential mechanism for androgen refractive prostatic growth. To investigate the potential contribution of Wnt in the TGF-ß-androgen receptor paracrine signaling effects, we treated Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} stromal cells with bicalutamide to quantitate Wnt ligands expressed as a result of androgen ablation over a time course of five days. We performed semi-quantitative RT-PCR on the two stromal cell populations to find an increase in Wnt-2, Wnt-5a, and Wnt 9a one to three days after bicalutamide treatment on Tgfbr2$^{floxE2/floxE2}$ stromal cells with a decrease in expression at five days (Figure 4). A similar trend was seen with Tgfbr2^{fspKO} stromal cells, however with greater basal expression for Wnt-2 and Wnt-5a. Thus inhibiting the androgen signaling pathway can induce Wnt gene expression in normal prostatic stromal cells, yet the loss of stromal TGF-ß responsivity results in a constitutive expression of the same genes.

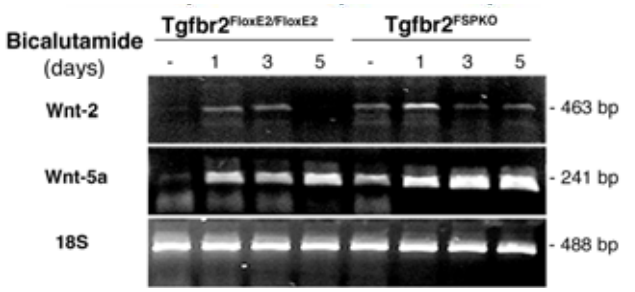

Figure 4. RT-PCR show that Wnt genes are expressed by control (Tgfbr2$^{floxE2/floxE2}$) stromal cells as a result of androgen receptor antagonism through the incubation with bicalutamide. The Tgfbr2^{fspKO} prostatic stromal cells express Wnt3a and Wnt5a constitutively. 18S expression was used as a loading control.

Next, to determine the potential epithelial ramifications of the bicalutamide induced Wnt ligands, the cognate receptor was antagonized through the expression of secreted frizzled related protein-2 (SFRP-2). When we infected our stromal (Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO}) and epithelial cells (PC-3, RWPE-1, DU145, and LNCaP) with SFRP-2 in the presence and absence of bicalutamide the proliferation was inhibited more than using bicalutamide alone (Figure 5). The combination of bicalutamide and SFRP-2 decreased Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} proliferation approximately 20% of untreated basal levels, but nearly inhibited all proliferation of the epithelial lines tested (appreciable cell death was not observed). The proliferative effect of bicalutamide treated Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} – conditioned media on LNCaP cells was nearly neutralized by the expression of SFRP-2. Thus stromally derived Wnt ligands can mediate the androgen refractile proliferation of prostatic epithelia.

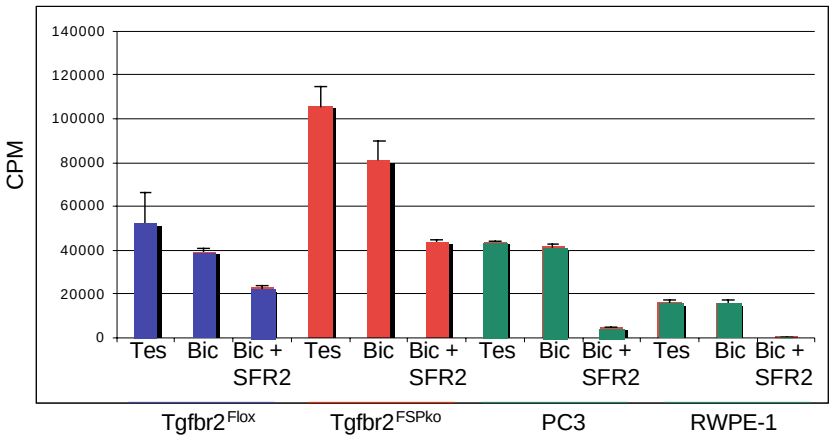

Figure 5. Thymidine incorporation assays of prostatic stromal and epithelial cell lines (PC3 and RWPE-1) suggest differential responsivity to androgen ablation through bicalutamide treatment. However, the combination treatment with SFRP2 and bicalutamide uniformly suppresses the proliferation of both androgen sensitive and insensitive cells.

Task 3

We have no evidence for the competition of E-cadherin and AR for the binding to ß-catenin *in vivo*. It seems that both can bind ß-catenin however, the exclusive of binding of AR or E-cadherin seems to be primarily based on cellular compartmentalization. E-caherin is found at the cytoplasmic membrane while AR is found in the nucleus in the presence of androgen or bicalutamide. However, it should be noted that ICAT, an inhibitor of TCF-4, and E-cadherin binding to ß-catenin also blocked binding of the androgen receptor [12]. However, in our search for factors that mediate the ß-catenin-AR interaction, we again looked to the differences in the expression of transcription factors between prostatic stromal fibroblasts and epithelia. The protein Hic-5/ARR-55 has been identified as a stromal factor that's expression is induced by TGF-ß and binds with AR [13, 14]. We uniquely identified Hic-5 to bind ß-catenin irrespective of ligand occupation of AR. The overexpression of Hic-5 in LNCaP cells resulted in decreased ß-catenin-mediated transcriptional activity. The paracrine role *in vivo* for the expression of Hic-5 was tested through stromal-conditioned media experiments were performed with

parental LNCaP cells and Hic-5 overexpressing LNCaP cells. The overexpression of Hic-5 did not significantly affect the proliferation of the LNCaP cells in culture, as determined by thymidine incorporation assays. As above (see Figure 4), $Tgfbr2^{floxE2/floxE2}$ stromal–conditioned media resulted in decreased proliferation of both parental LNCaP and Hic-5 expressing LNCaP cells. Strikingly, the $Tgfbr2^{fspKO}$ stromal-conditioned media resulted in proliferation of LNCaP cells, but not the Hic-5 expressing LNCaP cells. Similarly, Hic-5 expression allowed the LNCaP cells restored bicalutamide sensitivity in the presence of $Tgfbr2^{fspKO}$ stromal-conditioned media. Together, these results support an important role of canonical Wnt signaling in androgen responsivity of prostate cancer cells. Further, the expression of Hic-5 can enable hormonally refractive cells to become hormonally sensitive.

KEY RESEARCH ACCOMPLISHEMENTS

- Demonstrate AR can suppress ß-catenin signaling in the presence of DHT.
- Showed that the dynamic loading of ß-catenin and AR through re-ChIP experiments on the PSA enhancer and promoter regions.
- Prostatic stromal cells exhibit AR and ß-catenin interactions similar to prostatic epithelial cells with the exception of the inclusion of Hic-5 in stromal cells.
- Prostatic stromal cells from androgen-nonresponsive prostate tissue have differential expression of Wnt genes compared to control prostatic stromal cells.
- Wnt 3a and Wnt5a expression can cause otherwise androgen responsive LNCaP cells to become refractive to androgen ablation
- The expression of Hic-5 can restore androgen responsivity in LNCaP cells under conditions where androgen responsivity is lost.

CONCLUSIONS

Wnt-mediated canonical downstream signaling through ß-catenin transcriptional activity can negatively regulate androgen responsivity of prostatic epithelial cells. The prostatic stroma can be the source of the Wnt ligands in the context of androgen ablation, *in vivo.* Our results support the data that show ß-catenin mutations enabling ß-catenin stability in prostate cancer can lead to androgen independence. At least two genetic interventions for hormonally refractive prostate cancer have emerged from our work: 1) the expression of Hic-5 in the prostatic epithelia to inhibit canonical Wnt signaling and 2) antagonism of Wnt receptor/ligand interaction such as the expression of SFRP-2.'

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